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<b>(21) International Application Number:</b> PCT/US87/00711 <b>(22) International Filing Date:</b> 1 April 1987 (01.04.87) <b>(31) Priority Application Number:</b> 846,757 <b>(32) Priority Date:</b> 1 April 1986 (01.04.86) <b>(33) Priority Country:</b> US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 846,757 (CIP) Filed on 1 April 1986 (01.04.86)  <b>(71) Applicant (for all designated States except US):</b> GENEL-ABS INCORPORATED [US/US]; 505 Penobscot Drive, Redwood City, CA 94063 (US).		<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> FOUNG, Steven, K., H. [US/US]; 885 Allardice Way, Stanford, CA 94305 (US). RABIN, Linda, B. [US/US]; 866 Laurel Street, Alameda, CA 94501 (US).  <b>(74) Agents:</b> DEHLINGER, Peter, J. et al.; Ciotti & Murashige, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> IMMORTALIZED VIRUS-SPECIFIC TISSUE CELLS  <b>(57) Abstract</b>  An immortalized human tissue cell line formed by fusing a selected human, or non-human primate non-lymphocytic tissue cell with an immortalizing human/mouse hybridoma cell. The tissue cell, and the resulting fusion product cell line is infected or infectable by a selected viral agent, allowing virus-specific cell surface antigens to be identified, and used as a source for generating virus-specific antigens, anti-antigen antibodies, and vaccine compositions.		

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IMMORTALIZED VIRUS-SPECIFIC TISSUE CELLS10 1. Field of the Invention

The present invention relates to immortalized human or non-human primate cells which are infected or infectable by tissue-specific viruses, to methods for preparing the cells, and to uses of the cells in identifying virus-specific antigens and anti-antigen antibodies.

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### 3. Background of the Invention

For a number of important viral or suspected  
viral diseases in humans, it has proven difficult or  
impossible to identify the virus or viral agent of  
15 interest, and therefore research into understanding the  
nature of the virus and in developing diagnostic and  
therapeutic tools for treating and preventing infection  
by the virus has been limited.

One reason that many human-pathogen viruses  
20 have been difficult to identify and characterize has  
been the lack of a system for propagating the virus  
outside of a human host. This problem is illustrated in  
the case of hepatitis nonA nonB (NANB) virus, a major  
cause of post-transfusion hepatitis. An etiological  
25 NANB virus or viruses have been directly demonstrated by  
infecting chimpanzees (Bradley, Alter, Hoofnagle), but  
so far chimpanzees remain the only experimental animal  
system for studying the virus. The difficulty and  
expense in obtaining adequate amounts of virus-infected  
30 material have seriously limited research on this virus.  
Similar problems are encountered with the hepatitis B  
virus, which can be grown in chimpanzees and marmoset  
monkeys, but not in inexpensive lab animals or in cell  
culture.

Although existing cell culture systems can be used to propagate some viruses, this approach has not been successful for many viruses which are specific for certain cell types, particularly if long propagation times are involved. The reason for this limitation is that many normal human cells, such as hepatocytes, which are the specific targets of a virus of interest, such as NANB virus, cannot be propagated in culture, and can be maintained in culture for periods of usually only 1-2 weeks. For many viruses, either the cells in short term culture do not take up infecting virus efficiently, or the total incubation time required for viral propagation in the cells is greater than the period of cell viability. In any case, it has been impossible heretofore to propagate a number of important human-pathogen viruses, such as NANB or hepatitis B viruses, in normal human cells in culture.

Thus, tissue-specific human cells which could be grown stably in culture would provide a convenient, and in some cases, unique host for many human infectious viruses. This is true particularly for viruses which require tissue-specific surface antigens for cell infection, and which also have relatively long incubation times.

The ability to propagate certain normal human cell types in culture would also provide a tool for identifying viral agents which are suspected as causative agents of diseases of that cell type. Several diseases of the central nervous system, such as Alzheimer's disease and multiple sclerosis, are suspected of having a viral origin, and the ability to produce stable cultures of central nervous cells from patients with these diseases would provide a convenient source of virus-infected material which could be used

for studying and identifying virus-specific cell-surface antigens and viral genomes.

Stable, cultured human or non-human primate cells derived from a selected normal cell type would also be useful in studying metabolism and drug effects in specific cell types. Metabolic activity in many tissue specific cell types, including cells derived from liver, pancreas, pituitary, and brain tissue in short-term culture has been studied. These studies have been limited heretofore by the amount of work required to isolate cells for culture, and most studies have been confined to non-human cell cultures, for reasons of cell availability. Additionally, the length of the studies is necessarily limited to the one to several week period of cell viability. Such limitations would not apply to stable, tissue-specific cell types in culture. Further, the ability to infect the cells with tissue-specific viruses would open up new avenues for studying drug effects on infectious viruses.

Given the potential advantages of being able to propagate tissue-specific cells, particularly human and non-human primate cells, stably in culture, it is not surprising that considerable effort has been invested in this problem. To date, however, these efforts have been largely unsuccessful. Efforts to extend the culture period of cultured hepatocytes, such as by coculturing the liver epithelial cells, have produced only limited increase in culture times (Guguen-Guillouzzo). Some malignant cells associated with specific human tissues, such as hepatoma cells derived from malignant liver tissue, are able to grow stably in culture. However, cultured malignant cells are of limited use as a host system for tissue-specific viruses, both because the cells are often deficient in specific cell receptors

needed for viral infectivity, and because viral genomes are often associated with malignancies (Knowles), and therefore the identity of the infecting virus would be difficult to confirm.

5           Attempts to produce stable, tissue-specific cells in culture by fusing the cells with related myeloma cells have also been attempted. This approach is analogous to the one used for immortalizing  
10 antibody-secreting human lymphocytes, by fusion with myeloma cells under defined selection conditions. Heretofore no successful application of this technique have been reported.

#### 4. Summary of the Invention

15           It is therefore an important object of the invention to provide a method for producing human and non-human primate cells which are stable in culture and which are infected or infectable with tissue-specific human-pathogen viruses.

20           A related object is to provide an immortalized human or non-human primate cell line which is infected or infectable with a selected tissue-specific virus.

          Another object of the invention is to provide methods of using virus infected immortalized cells to  
25 identify and obtain virus-specific cell-surface antigens, monoclonal antibodies specific against such antigens, viral genomic material and/or viral antigens useful in producing anti-virus vaccines.

          Still another object of the invention is to  
30 provide virus-specific cell surface antigens which are produced with viral infection of human cells, and diagnostic methods which use the antigens to detect the presence of virus-specific antibodies in the serum of a human infected with the viral agent.

The invention includes immortalized non-lymphocytic, non-malignant human or non-human primate cells derived from a selected, non-lymphocytic tissue. The cells are formed by fusion of a mouse/human hybridoma with a human or non-human primate cell derived from the selected non-lymphocytic tissue. The tissue-specific cell is one which is infectable or infected with a selected viral agent in vivo, and may include, for example, hepatocytes, central nervous system cells, and synovial cells. The fusion partner is produced by fusing mouse myeloma cells and human B-lymphocytes, and selecting fusion products which show stable human chromosome retention, as evidenced by continued HLA surface antigen production in culture. The fusion partner is fused with the tissue-selected cell under conditions which allow growth of successful trioma fusion products only.

In a preferred embodiment of the invention, the fusion partner is formed by fusing mouse myeloma cells and human B-lymphocytes, selecting fusion products which show immunoglobulin secretion and HLA surface antigen production in culture, and treating the selected fusion products with a mutagen. The mutagenized fusion products are selected for those which retain the ability to produce HLA surface antigen, show no immunoglobulin secretion, and are unable to survive in a growth medium which allows growth of a successful product formed by fusing the fusion partner with such a human cell. An exemplary fusion partner has the characteristics of ATCC number HB 8464.

The immortalized cell line, when infected with the selected virus, can be used to identify virus-specific cell-surface antigens, as a source of such antigens, for identifying virus-specific

antibodies, and/or for identifying viral genomic material which can be used as a probe for detecting the viral agent, or for producing virus-antigen peptides for vaccine purposes.

5           These and other objects and features of the invention will become more fully apparent from the following detailed description of the invention.

Detailed Description of the Invention

10

I. Definitions

          As used herein, "trioma" refers to a cell line which contains genetic components originating in three originally separate cell lineages. As used in the  
15       context of this application, these triomas are stable, immortalized cells which result from the fusion of a murine myeloma/human hybridoma with a non-lymphocytic tissue cell from a human or non-human primate source.

          The murine myeloma/human hybridoma (the  
20       "immortalizing hybridoma" or "fusion partner") is an immortal cell line which results from the fusion of a murine myeloma or other murine tumor cell with human lymphoid cells derived from a normal (preferably non-immunized) subject. As described below, by careful  
25       selection and mutation, an immortalizing hybridoma which provides improved chromosomal stability, has human characteristics, and which does not secrete immunoglobulin is obtained.

          "Non-secreting" hybridoma refers to a hybridoma  
30       which is capable of continuous reproduction and, therefore, is immortal but lacks the capacity to secrete immunoglobulin.

          A hybridoma "having human characteristics" refers to a hybridoma which retains detectable

human-derived chromosomes, such as those producing human HLA antigen which will be expressed on the cell surface.

"Tissue-specific cells" refer to non-lymphocytic human or non-human primate cells derived from a selected tissue or organ source, such as liver, the central nervous system or synovial fluid, and which are infected or infectable with a tissue-specific virus in vivo.

"Cell line" refers to various embodiments including but not limited to individual cells, harvested cells, and cultures containing cells so long as these are derived from cells of the cell line referred to. By "derived" is meant progeny or issue. It is further known in the art that spontaneous or induced changes can take place in karyotype during storage or transfer. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants.

## II. Preparing Stable Human and Non-Human Primate Cell Lines

### A. The Immortalizing Fusion Partner Cells

The cells which make up the immortalizing hybridoma are murine myeloma cells and human lymphoid B cells. Murine myeloma cell lines are commonly available and may be obtained through the American Type Culture Collection (ATCC), located at the National Institutes of Health (NIH) in Bethesda, Maryland. Human lymphoid B cells are isolated from the blood of normal individuals using conventional techniques. Such procedures include density gradient purification and separation of B cells from T cells using standard sheep erythrocyte rosetting.

### B. Tissue-Specific Cells

The tissue-specific cells which are to be immortalized by fusion with the mouse/human fusion partner are isolated cells derived from a selected, non-lymphocytic tissue from a human or non-human primate. The latter source includes primates, such as chimpanzees, whose phylogenetic similarity to humans (Clark) allows for successful fusion with the mouse/human fusion partner. The ability of the mouse/human fusion partner to fuse with and immortalize chimpanzee cells forms the basis of an immortalized chimpanzee cell line described in a co-owned patent application for "Non-Human Primate Monoclonal Antibodies and Methods", Serial No. 767,213, filed May 1, 1985.

The cells are isolated from a tissue or organ which is infectable or infected with a selected virus of interest. The isolated cells must also be capable of limited viability in culture, without appreciable loss of the desired tissue-specific property, for a short period until the cells have been successfully immortalized by fusion with the immortalizing partner.

A variety of methods for preparing tissue cells for culture have been developed, and these methods are generally suitable for isolating tissue-specific cells for fusion with an immortalizing mouse/human hybridoma according to the present invention. The following cell-preparation methods for specific cell types are illustrative of general techniques which are applicable.

Several methods for producing isolated hepatocytes for cell culture have been reported (Krack, Guguen-Guillouzzo). In general, these methods involve perfusing liver tissue with a solution of collagenase, mincing the enzyme-digested tissue to release free cells, and filtering the isolated cells through a cloth

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mesh. A simpler method of cell preparation which does not involve collagenase treatment has been implemented by the inventors, and has been shown to give good fusion efficiency with the immortalizing partner. In this method, the isolated tissue, such as fetal abortus liver tissue, is simply homogenized in a ground glass homogenizer having a single cell clearance, and the homogenized cells washed several times in a suitable culture medium, as described in Example II. Similar methods have been used to produce short-term cell cultures of pancreatic islet cells (Bone), anterior pituitary cells (Liang, Loughlin), and primary pituitary cells (Ben-Jonathon).

Methods for preparing cells derived from the central nervous system for cell culture have been demonstrated for a variety of CNS cell types, including spinal cord neurons (Kato), neurons from human cerebral cortex (Louis), and human glial cells (Ponten). As indicated above, immortalized nerve cells are likely to be used primarily in studying neurological disorders which are suspected of having viral origin. Therefore, the nervous-system cells which are isolated for immortalization, according to the method of the invention, will typically be derived from human or non-human primate subjects which have been positively diagnosed as having the disease under study.

Methods for preparing human synovial cells, including macrophages and fibroblasts, have also been reported (Cunningham, Kouri, Clarris). Where the synovial cells are used to study viral etiology in arthritis, the cells are of course obtained from patients suspected of having a virus-related arthritic condition.

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### C. Fusion Procedures

Fusions to form the murine-human non-secreting hybridomas and the immortalized tissue cell lines of the invention are performed by a modification of the method of Kohler and Milstein . Briefly, a mouse myeloma and human lymphocyte (to make the immortalizing hybridoma) or the hybridoma and the isolated tissue-specific cell (to make the immortalized tissue cell line) are combined in the presence of a fusogen such as polyethylene glycol under suitable conditions, e.g., 40%-50% polyethylene glycol (1000 to 4000 molecular weight) at between room temperature and 40°C, preferably about 37°C. Fusion requires about 5-10 minutes, and the cells are then centrifuged and screened.

Alternatively, fusion products, such as the fusion of the immortalizing fusion partner and the human non-lymphocyte tissue cell, can be produced successfully by electrofusion, using known procedures.

### D. Screening Procedures

Following the fusion procedure, screening for hybridized products is made by culturing cells centrifuged from the fusion medium in growth medium which is selective for the desired hybrids. Ordinarily, non-immortalized cells cannot survive repeated transfers on any medium, and hence will not survive repeated culturing of the centrifuged cells. Commonly used lines of immortalized murine myeloma cells, however, are incapable of growth on certain selective media which have been chosen to deprive them of their ability to synthesize DNA. Two very commonly used media of this description are "hypoxanthine-aminopterin-thymidine" or "HAT" medium and azaserine-hypoxanthine medium or "AH" medium.

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Both of these selection media take advantage of the capacity of normal cells to utilize a "salvage" pathway for DNA synthesis under circumstances where the de novo process is inhibited. Aminopterin inhibits both  
5 de novo purine and pyrimidine nucleotide synthesis in normal cells and both thymidine and hypoxanthine are required for the salvage pathway. Azaserine inhibits only purine synthesis, so only hypoxanthine is required for the salvage pathway.

10 The salvage process, which requires hypoxanthine phosphoribosyl transferase (HPRT) is generally inoperable in the commonly used murine myeloma cells (although they retain the de novo pathway). Since aminopterin (in the HAT medium) or azaserine (in  
15 the AH medium) are both inhibitors of the de novo DNA synthesis pathway, the murine myeloma cells are incapable of growth in either "HAT" or "AH" medium. Thus, only hybridized cells can both survive repeated transfers and grow in HAT or AH medium. Normal  
20 lymphocytes cannot survive because they are not immortalized, and do not survive repeated transfers; unhybridized myeloma cells cannot survive because they lack the salvage pathway which permits the use of hypoxanthine to overcome aminopterin or azaserine  
25 inhibition.

#### E. The Immortalizing Hybridoma

The selection procedures employed in producing the immortalizing hybridoma are aimed at selecting cells  
30 which show stable human characteristics, non-secretion of immunoglobulin, and sensitivity to a defined selection medium which can be used for selecting successful immortalized tissue cell hybrids. Briefly, the cells centrifuged from the fusion mixture of mouse

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myeloma and human lymphoid cells are diluted and plated in microtiter plates. Screening is done using AH or HAT medium growth, with selection of successful colonies being made on the basis of assay procedures related to stability and human character. From among the many colonies assayed, several are chosen which continue to produce immunoglobulin in the supernatant fluid for a suitable period of time, preferably in excess of six months (one criterion for stability). Here it is noted that the culture period of two-three months required to obtain a sufficient number of cells for mutagenesis, as described below, may also serve as a period for gauging stability of immunoglobulin secretion. The continued production of such immunoglobulin indicates that the characteristics conferred by the human lymphocyte partner have not been lost (lymphocytes which were unfused will, of course, not survive). Retention of human characteristics is assessed by assaying the cell surfaces for the presence of HLA antigen. The selected colonies continue to exhibit HLA antigen expression at their cell surfaces, and in fact, continued production of immunoglobulin and HLA surface antigen appear to be linked, as the two traits are invariably found in the same cells.

The selected cells are then treated with a mutagen, such as 6-thioguanine, to destroy their ability to secrete immunoglobulin and confer HAT or AH sensitivity. This will make possible later fusion to give an immortalized tissue-specific cell, and subsequent use of the fusion product for protein secretion, without a contribution of immunoglobulin secretion from the fusion partner. The mutagenized cells are also selected for retention of HLA antigen expression on the cell surface.

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Example I below describes the preparation of an immortalizing hybridoma which is generally useful in producing primate triomas according to the invention. The cell line, designated SBC-H20, has the selected characteristics noted in the example. The cell line was deposited with the ATCC on or about 13 December 1983 and given the designation ATCC HB 8464.

F. Immortalized Human or Non-Human Primate Cell Line

Human or non-human primate tissue cells from above are fused under conditions like those outlined in Section IIC. Typically, the tissue cells are mixed with the immortalizing hybridoma cells at a ratio of between about 1:1 to 1:5, and preferably about 1:3. Here it is noted that cell mixtures containing greater numbers of the tissue cells, such as in a ratio of 1:1 and 5:1 are feasible. However, since the tissue cells are usually more difficult to obtain, it is generally preferred to use an excess of the immortalizing cells in the cell fusion mixture. The cell mixture is freed of serum by washing, and resuspended in polyethylene glycol to promote cell fusion. After a suitable incubation period, the cells are washed, resuspended in culture medium, and plated on microtiter wells. Since the unfused tissue-specific cell is unable to grow in culture, the selection medium can simply be the HAT or AH medium which will discriminate against unfused immortalizing hybridoma cells. The resulting fusion products are thus easily selected on the basis of the presence of colonies of growing cells, as seen by light microscopy.

### G. Hybrid Cell Line Selection

In addition to the selection of hybrid cells based on their ability to grow in HAT or AH medium, it is generally advantageous, prior to viral infectivity studies, to confirm that the hybrid cells contain selected characteristics of the tissue cell type which has been fused. In the case of cells which are derived from secretory tissue, such as liver, one convenient confirmatory assay involves testing the hybrid cell for the ability to secrete one or more selected tissue-specific secretory proteins. The assay can be carried out by means of a solid phase reporter-linked immunoassay. In the usual solid-phase assay method, a solid surface coated with antibodies specific against the selected protein is reacted with the cell culture supernatant, binding supernatant proteins immunospecifically to the support surface. Because of the close structural relationship between human and primate (particularly chimpanzee) secretory proteins, antibodies which are specific against selected human secretory proteins, such as human serum albumin (HSA) may also be used to screen for corresponding non-human secretory proteins. Purified goat or rabbit serum antibodies against a variety of human secretory proteins and peptide hormone, such as serum albumin, are commercially available. Also mouse monoclonal antibodies against many human secretory proteins are available.

Where a protein-specific antibody is not commercially available, the antibody can be produced by conventional serum antibody techniques for raising antibodies in rabbits or goats. These procedures generally require a source of purified or nearly purified protein for animal inoculation. Procedures

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for purifying many human secretory proteins have been reported, and are documented below. Alternatively, conventional monoclonal antibody techniques may be used for generating antibodies against selected human proteins.

After reacting the cell medium with the support-bound antibodies, to bind antibody-specific secretory protein, the support is washed to remove non-specifically bound protein and then reacted with a soluble, reporter-labeled antibody which is also specific against the secretory protein of interest. The reporter label on the soluble antibody is a fluorophore, chromophore, enzyme, or radioisotope label. Techniques for labeling antibodies with reporter molecules are well known, and typically involve the use of a bifunctional coupling reagent, such as a di-N-hydroxysuccinimide, or a suitable carboxyl or amine activating agent, such as a soluble carbodiimide, to couple one or more reporter molecules to the antibody. Such methods are well known to those in the field. The labeled antibody may be specific against the same or a different antigenic determinant as the support-bound antibody, but in any case, must be able to bind to the selected protein, with such bound immunospecifically to the first antibody carried on the solid support. The presence of the selected protein in the cell medium is confirmed by the presence of label on the washed support. Example III below illustrates an enzyme-linked immunoassay (ELISA) for detecting human serum albumin (HSA), complement C<sub>3</sub> (C<sub>3</sub>), and human fibronectin (HFN) secreted by immortalized liver.

Alternatively, where the cell type which is immortalized does not have secretory functions, other cell-specific characteristics, such as the presence of

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surface antigens or cell morphological characteristics can be used to confirm that the hybrid cell line is composed of the cell line of interest. Cell surface antigens can be readily assayed, according to procedures  
5 which will be detailed below, by providing an antibody which is specific against a selected tissue-type antigen, labeling the antibody with a suitable reporter, reacting the labeled antibody with the cells, and detecting the presence of reporter on the cells, after  
10 washing.

After identifying one or more viable hybrid cell colonies which secrete the protein of interest, the cells can be subcloned to ensure monoclonality. Typically, subcloning is done by a limiting dilution  
15 technique in which parent hybrid cells are diluted to produce no more than about 1 cell per well, and the cells replated in multi-well microtiter plates. Clonal populations which show the desired tissue-specific property are then isolated.

20 Experiments conducted in support of the present invention, and reported particularly in Example III, demonstrate that human non-lymphocytic tissue cells, such as human liver cells, can be immortalized so as to be capable of continued replication in culture over a  
25 period of at least several months, and at the same time, retain tissue-specific cell functions, such as secretion of one or more liver-specific proteins. The results are consistent with earlier studies by the inventors and their colleagues showing that the mouse/human fusion  
30 partner described herein is capable of immortalizing human and non-human primate lymphocytes, to produce cells which are stable in culture for periods of up to several years, with little diminishment in secretory activity. For both lymphocytic and non-lymphocytic cell

types, the fusion process is characterized by a high efficiency of successful hybrids, and of these, a high percentage of hybrid cells which are active and stable in secreting tissue-specific proteins.

5           The high efficiency of cell fusion, for both lymphocytic and non-lymphocytic cells, appears to be related to the initial selection, in forming the fusion partner, of mouse/human hybrids which are stable for human chromosome retention, as evidenced by continued  
10 immunoglobulin secretion and HLA production. That is, the fusion partner is preselected for a stable arrangement of human and mouse chromosomes, which apparently favors chromosome stability in the trioma fusion product. The ability of the fusion partner to  
15 form stable fusion products with such diverse cells as human lymphocytes, chimpanzee lymphocytes, and human liver cells indicates the general applicability of the method to human cell immortalization.

20 G. Viral Infectivity

          According to one important aspect of the invention, the immortalized cells are used as an infectable host for certain human or non-human primate viruses, and particularly those which can not otherwise  
25 be grown in culture. Here parent hybrid tissue cells, such as fused liver cells, are selected for the presence of colonies, and the parent cells are then assayed for infectability by the virus. Typically, virus infection is detected by the steps of (a) infecting the cells with  
30 a known source of the active virus and (b) assaying the cells during a one to several week incubation period, for the presence of viral infection. Initially, for identifying parent hybrid cells which are susceptible to viral infection, the trays containing the fused cell

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colonies are duplicated, so that a stock of uninfected cells will be available after the virus-infected cell colonies are identified.

5 The virus-induced cell change is preferably one which is readily and unambiguously detected in the culture of immortalized tissue cells. The viral infection may be characterized by cytopathic effects (CPE), such as cell lysis, multinucleation or cell clumping. Other types of virus-induced cell responses  
10 included metabolic changes, such as a pH shift or release of one or more cellular enzymes. Both of these types of metabolic changes can be detected by the use of suitable indicator dyes or substrates.

15 Finally, and according to one important example of the invention, viral infection can be determined by the presence of virus-specific surface or intracellular or extracellular antigens which are expressed as a result of viral infection. In general, cell surface antigens can be detected readily by conventional labeled  
20 antibody techniques. In one approach, the cultured cells are first reacted with the serum from a human or non-human primate infected with the virus. After washing the cells to remove non-specifically bound material, the cells are incubated with a  
25 reporter-labeled anti-human antibody, preferably a fluorescent-labeled antibody. Infected cells are then easily detected by the presence of reporter on the cell surface. This method is illustrated in Examples V and VI for detecting immortalized cells infected with NANB  
30 hepatitis virus.

A specific liver hybrid cell which has been infected with NANB virus from an infected chimpanzee, and which shows virus-specific cell surface antigens has been deposited with the American Type Culture

Collection, and is identified by ATCC number HB 9027.  
The hybrid liver cell which is infectable with NANB from  
an infected chimpanzee is also infectable with NANB from  
human plasma, and with hepatitis B virus from an  
5 infected human, also as described in Example V.

### III. Uses of Virus-Infected Hybrid Cells

#### A. Viral Propagation

10       As described above, and illustrated in Example  
V, hybrid cells formed in accordance with the invention  
can be infected with, and used to propagate  
tissue-specific viruses which are otherwise not capable  
of being propagated in culture. The general methods for  
15 infecting and culturing the hybrid cells with a selected  
human-infectious virus generally follow the procedures  
outlined above and described in Example V. Briefly,  
plasma from a human or other infected source, such as  
chimpanzee, is used to infect the cells, and viral  
20 infection is followed by monitoring a virus-related cell  
change over time in culture. Where virus infection is  
characterized by the appearance of virus-specific  
antigens, as in the case of the two hepatitis viruses  
propagated as in Example V, the viral infection is  
25 preferably followed by immunological methods for  
detecting antigens. After viral infection and  
propagation, the virus can be harvested, if desired, by  
conventional methods for releasing and purifying viral  
particles from cells.

30       The infectability of the cells, and the ability  
of serially passage a viral agent from infected to  
non-infected cell line is demonstrated in Example VI.  
Here each of two immortalized liver cell lines infected  
with NANB viral agent were cultured under normal growth

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conditions, then lysed by one of a variety of means to release a supernatant fraction. The supernatant fractions (or mitomycin-treated cells) were added to a non-infected immortalized cell line and the  
5 newly-infected cells were followed over a several week period for NANB-specific cell surface antigens. In all cases, the infected cells showed immunofluorescent reactivity (surface antigen appearance) within ten weeks of infection. The supernatant from a second generation  
10 infected cell line was similarly passaged to uninfected cells in culture, yielding infected third generation cells.

B. Identification of Antigens Related to Viral Infection

15 As indicated above, viral infection of the hybrid cells is often accompanied by cell expression of antigens which are specific to the viral infection. The antigens may be intracellular, bound to the surfaces of the infected cells, and/or expressed in extracellular  
20 form. One general method for determining the presence of virus-specific antigens, which is outlined above and illustrated in Example V, involves immunospecific binding between the antigen and anti-antigen antibody from the serum of a human or experimental animal which  
25 has the infection. This method of course depends on the presence of such antibodies in the infected subject. In the case of cell-surface antigens, the assay typically involves reacting the serum with the cells, washing the cells to remove unbound serum components, then reacting  
30 the cells with a labeled anti-immunoglobulin antibody which can bind specifically with the anti-antigen antibody. This approach is detailed in Example V. Intracellular and extracellular antigens can be assayed by conventional immunoassays which may involve

immunoprecipitation or Ouchterlony immunodiffusion techniques, using the above serum and a cell-free antigen preparation. Experiments conducted in support of the present invention have shown that hybrid liver  
5 cells which are infected with hepatitis B virus express hepatitis B surface antigens which are immunoreactive with a well-defined antibody preparation to hepatitis B surface antigen.

The detection and partial isolation of a  
10 virus-specific surface antigen, in response to infection with NANB viral agent, is illustrated in Example VII. Two NANB-infected cell lines, designated RO2 and RS1, and one uninfected line, designated GL424, were examined for the presence of plasma membrane antigens which react  
15 specifically with antibodies from a NANB-infected individual. Plasma membrane fractions of the three cell lines were prepared by the methods described below, and the three membrane fractions were dot blotted onto filters by conventional methods. The dot blots were  
20 reacted with IgG antibody fractions obtained from either normal or NANB-infected individuals, then examined for antibody binding by addition of a fluorescent-labeled anti-human IgG antibody. The results are given in Table 4 of Example VII. At concentrations of membrane protein  
25 between about 1 and 10 µg/well, both of the infected cell lines gave greater immunoreactivity with NANB(+) IgG fraction than with the normal fraction, demonstrating that the antigen fraction can be used to assay for NANB infection, based on the presence of  
30 NANB-specific antibodies which are present in the infected individual and reactive with the fraction.

More generally, the method allows for detection of infection in humans of a viral agent which provokes a virus-specific antibody response on infection. The

method involves isolating from immortalized tissue cells which are infected with the viral agent, a fraction containing a cell surface antigen which shows greater immunological reactivity with antibodies obtained from  
5 an infected individual. The fraction is reacted with a blood sample, e.g., a serum sample or IgG fraction, from the test individual, and the binding of antibody to the antigen is detected. A typical assay procedure follows the method in Example VII, where analyte human serum is  
10 reacted with the cells, and the cells are exposed, after washing, to a labeled anti-human immunoglobulin antibody.

This general approach may also be suitable for detecting the presence of viral infection of certain  
15 immortalized cell types, such as central nervous system cells, where viral etiology is suspected. Here the immortalized cells are exposed to serum derived from the cell donor or another individual having the disease, with the presence of cell antigens being determined by a  
20 second labeled antibody, as above. As in all of the antigen-specific tests, the specificity of the antigen must be confirmed by suitable controls involving an immortalized control (non-infected cells).

25 C. Producing Virus-Specific Monoclonal Antibodies

The virus-specific antigen from above, either in cell-bound or soluble form, is also useful for generating virus-specific murine monoclonal antibodies (Mabs). The method follows standard Mab procedures, in  
30 which a mouse is inoculated with cell or cell-free material containing the antigen, and B-lymphocytes obtained from the inoculated animal are fused with mouse myeloma cells, according to fusion procedures like those detailed above. Successful fusion products can be

determined by assaying microtiter wells for the presence of the desired Mab, using assay techniques similar to those for detecting serum antibodies which are specific against the antigen.

5           In one application, the Mabs are useful in diagnosing virus infection, where infection is evidenced by the presence of the virus-related antigen in the serum of the infected individual. This assay can be performed, for example, as a solid-phase sandwich-type  
10 assay, in which the antigen is first reacted with the Mab attached to a solid support, followed by attachment of a soluble labeled anti-antigen Mab to the support through the antigen.

          Another application of the Mabs is for  
15 purifying the virus-specific antigen, using conventional affinity chromatography techniques. The purified antigen would be valuable, for example, in designing an anti-virus vaccine, based on the known amino-acid sequence of the purified antigen.

20

#### D. Detection of Active Viral Genes

          An important advantage of the present invention is the ability to propagate relatively large amounts of viral genomic material in the hybrid cell cultures. The  
25 genomic material may be either in the form of self-replicating viruses, or, if the viral genome is integrated into the cell genome, as part of the replicating cell DNA. In either case, the presence and identity of active viral genes can be determined by DNA  
30 subtraction enrichment methods which have been reported (Kunkel), or by antigen expression in a suitable expression system. The latter method is performed, for example, by preparing a cDNA library of polyA RNA isolated from the virus-infected cells, cloning the

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library fragments in a suitable cloning vector, transforming host cells, typically a bacterial host, with the vector, and using the anti-antigen antibody described above to detect the expression of the antigen  
5 by the host.

The identified viral genomic material may be used as a probe for identifying further the presence and or localization of viral genome in the infected cell, as a diagnostic tool for detecting the presence of virus in  
10 a blood or tissue sample from an individual, or in molecular cloning procedures, for producing antigen or antigen fragment peptides which are useful for vaccine purposes.

The following examples illustrate various  
15 aspects of the invention, but are in no way intended to limit the scope thereof.

#### Example I

##### Preparation of Immortalizing Hybridoma SBC-H2O

20 Mouse myeloma cell line SP20/08A2 was obtained for use as the immortalizing partner from Frank Fitch, University of Chicago. This cell line is freely available and can be used without restriction. Other mouse myeloma lines are also readily available. Human  
25 peripheral B lymphocytes were isolated from the heparinized blood of a normal human donor by Ficoll-Hypaque gradient as described in reference 11. The peripheral B lymphocytes and myeloma cells were mixed at a 1:1 ratio, washed once in RPMI 1640 medium  
30 (Gibco), and pelleted at 250 x g for 10 min. The pellet was gently resuspended in 1 ml of RPMI with 40-45% (V/V) polyethylene glycol solution, MW 1430-1570 (BDH Chemicals, Poole, England) which was pre-warmed to 37°C. After two min at room temperature, the cell

suspension was diluted to 6 ml with RPMI, centrifuged at 500 x g for 3 min, and, beginning 8 min from the onset of fusion, the cell pellet was washed with RPMI containing 10% fetal calf serum (FCS). The pelleted  
5 cells were plated in multi-well trays using suitable dilutions to obtain individual clones. The colonies were grown on AH selection medium containing 2 µg/ml azaserine and 100 µM hypoxanthine, and successful clones were assayed for immunoglobulin production and  
10 for HLA surface proteins using the assay methods described in reference 6.

A hybrid clone which was stable for immunoglobulin production, and which was consistently producing HLA surface protein, was selected.

15 This clone was placed in Iscove's medium (IMDM) (Gibco) containing 10% FCS, 2 mM glutamine, 100 units penicillin, 100 mg streptomycin per ml, as well as the mutagen 6-thioguanine (Sigma, St. Louis, MO). The concentration of 6-thioguanine was progressively  
20 increased to  $2 \times 10^{-5}$  M over a period of approximately 30 days. The resultant mutant hybrids were subcloned, and the colonies tested for immunoglobulin secretion. A non-secreting subclone which was HAT/AH sensitive, resistant to  $10^{-6}$  M ouabain, and which retained the  
25 ability to produce HLA surface antigen was selected. A sample of this cell line which is designated SBC-H20 was deposited with the ATCC and has the deposit identifying no. ATCC HB 8464. The characteristics of this murine-human hybridoma include: sensitivity to HAT and  
30 AH media, resistance to ouabain (Sigma) to a concentration of  $10^{-6}$  M, non-secretion of immunoglobulins, human chromosomal stability over time, and production of HLA surface protein.

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Example IIIsolation of Human Hepatocytes

Human fetal liver tissue was obtained from a therapeutic abortus and placed in Iscove's Medium (IMDM) with 20% fetal calf serum (FCS). The tissue was dispersed mechanically by several gentle strokes in a ground glass homogenizer with a single cell clearance. The cells were washed three times, with centrifugation, in IMDM medium without FCS.

10

Example IIIPreparing Immortalized Liver Cells

Isolated hepatocytes from Example II were suspended to a final cell concentration of about  $3 \times 10^7$  cells/ml in IMDM. The isolated cells were mixed with the hybridoma cell line SBC-H20 (Example I), at a cell ratio of about 3:1. The cells were washed three times in IMDM without serum, with pelleting at  $200 \times g$  for 10 minutes, and resuspended gently in 1 ml of 55% IMDM:45% polyethylene glycol (v/v) MW 1430-1570 (BDH Chemicals, Poole, England) which was prewarmed to  $37^\circ\text{C}$ .

The fused cells were resuspended in IMDM containing 10% FCS and  $100 \mu\text{M}$  hypoxanthine,  $19 \mu\text{M}$  thymidine (HT medium) and plated in microtiter wells at  $1 \times 10^5$  cells/well and in 24 well trays, at  $1 \times 10^6$  cells/well. Cultures were grown in a humidified incubator at  $37^\circ\text{C}$  in 6%  $\text{CO}_2$ . After 24 hours, the medium was changed to the selection medium consisting of HT medium with 800 nm aminopterin (HAT medium). The HAT selection medium was used for 14 days prior to switching to HT medium, at which point unfused SBC-H20 cells and unfused hepatocytes were 100% nonviable, as evidenced by trypan blue inclusion.

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Hybrid cells formed by fusion of the SBC-H20 fusion partner with the hepatocytes were observed in 49 of the 120 microtiter wells (Group A) and 45 of the 48 wells in the 24-well trays (Group B), as evidenced by the presence of colonies which were visible by light microscopy. Some of the wells or trays contained two or more distinct colonies.

The wells or trays containing visible colonies were tested for ability to secrete human serum albumin (HSA), complement C<sub>3</sub> (C<sub>3</sub>), and fibronectin (HFN), as evidenced by the presence of one or more of these liver secretory proteins in the culture medium. The enzyme-linked immunoassay used to detect the presence of the proteins is described in Example IV below. The results obtained for the 45 fused cell colonies in Group A, and the 49 fused cells in Group B are shown in Table I. As seen, several of the fused cell lines were active in secretion of one or more of the liver proteins, and 5 colonies in Group A and 8 colonies in Group B secreted all three proteins.

From these two groups, selected hybridomas were cloned by limiting dilution to ensure monoclonality. Multiple monoclonal cell lines were isolated which continued to secrete HSA, complement C<sub>3</sub>, and HFN.

Table 1

	<u>Parent hybrids*</u>	<u>Number</u>	<u>Albumin</u>	<u>Comple- ment</u>	<u>Fibro- nectin</u>	<u>All three factors</u>
30	Group A	45/48	8	17	20	5
	Group B	49/120	14	20	9	8

\*Supernatants from hybrids cultured in 24-well trays (Group A) and in microtiter trays (Group B).

Example IVIdentification of Liver Cell Products

Selected hybridomas from the Group A and Group B colonies in Example III were subcloned by limiting  
5 dilution to insure monoclonality, and reassayed for parenchymal secretory proteins. Four parent hybrid cell lines, designated B3, D4, D5, and D8, were subcloned, and each parent gave multiple monoclonal sublines, as indicated in Table II below. Each of the subclonal  
10 lines was assayed for the cell secretion of HSA,  $C_3$ , and HFN by the following enzyme-linked immunoassay.

Goat anti-human antibodies specific against human albumin, complement  $C_3$ , and fibronectin were obtained from Cappel Labs (Malvern, PA). Microtiter  
15 trays were coated with one of the three different anti-sera overnight with 50 nl/well of a 1:1000 dilution (1  $\mu$ g/ml) at 4 C in a moist chamber. The following day, 150 nl of a 0.2 % gelatin solution were added to each well for one hour at room temperature, to saturate  
20 non-specific binding sites in the wells. The trays were then washed four times with cold PBS /0.05% Tween-20.

Analyte and control samples (50  $\mu$ l) were added to the trays and incubated for 1 hour at room temperature. The analyte samples were undiluted medium  
25 obtained from the growing monoclonal cultures. The control samples contained human plasma diluted 1:20,000 (complement control), purified human fibronectin at 5  $\mu$ g/ml, and human serum albumin, at 25  $\mu$ g/ml. After incubation, the trays were washed three times with the  
30 above Tween solution.

Peroxidase-conjugated goat antibodies against human  $C_3$ , albumin, or fibronectin were purchased from Cappel Labs. The peroxidase-conjugated antibody (50  $\mu$ l), at a final dilution of about 1  $\mu$ g/ml antibody

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protein, was added to each of the wells, and incubated for one hour at room temperature, to bind the conjugated antibody to analyte secretory protein in sandwich fashion. After washing three times with the above Tween solution, substrate mixture was added and the peroxidase enzyme reactions were allowed to develop for about thirty minutes at room temperature before arresting the reaction by the addition of 100  $\mu$ l of 10% SDS. The substrate mixture was prepared by mixing 7.2 ml of a 0.1M citrate/HCL buffer, pH 4.2, 4.8 ml of 2.5 mM ABTS, and 80  $\mu$ l of a 1 M  $H_2O_2$  solution. The mixture was prepared about 30 minutes prior to addition to the washed trays. The assay mixture/SDS solutions were read 415 or 405 nm. As seen from the results in Table II, each of the parent hybrids B3, D4, and D8, (all HFN secretors) yielded multiple subclones which were also competent in HFN secretion only. Similarly parent hybrid cell line D4, which was active in producing all three liver proteins, yielded multiple subclones, each of which was competent in secreting all three proteins assayed.

Table 2

25	<u>Parent hybrid</u>	<u>Number</u>	<u>Albumin</u>	<u>Complement</u>	<u>Fibronectin</u>
	B3	3	0	0	3
	D4	9	2	3	9
30	D5	8	0	0	8
	D8	3	0	0	3

Example VInfection of Immortalized LiverCells With NANB or Hepatitis B Virus

Hybrid liver cells prepared as in Example III  
5 were subcloned by limiting dilution and a subclonal  
colony which secreted HFN was isolated. The hybrid  
cells were plated at  $1 \times 10^6$  cells/well in a 24-well  
tray and overlaid with 100  $\mu$ l of plasma from one of  
the following three sources: (a) chimpanzee plasma known  
10 to contain NANB viral agent(s) by passage into a second  
chimpanzee, (b) human plasma from an individual with  
acute post-transfusion NANB hepatitis, and (c)  
chimpanzee plasma known to contain hepatitis B virus.

After the initial incubation of the chimpanzee  
15 serum and cells, 0.5 ml of growth medium containing IMDM  
and 20% FCS was added to each well and the cells were  
grown at 37 C in a humidified 7% CO<sub>2</sub> incubator. The  
cultures were fed with the growth medium every 3-4 days,  
and liver hybrid cells were removed every week to assay  
20 for the presence of NANB or hepatitis B antigens. In  
the assay procedure, an aliquot of the culture  
containing about  $1 \times 10^7$  cells were removed from a  
well and pelleted by centrifugation at 200 x g for 10  
minutes. After washing the cells three times with PBS,  
25 the cells were resuspended to  $2.5 \times 10^6$  cells/ml, and  
10  $\mu$ l of the cell suspension was dropped on a  
microscope slide and allowed to air-dry. The dried  
cells were then fixed on the slide by addition of  
acetone for one minute. To minimize non-specific  
30 binding, the slides were preincubated with normal goat  
serum (1:10) for 30 minutes at room temperature in a  
moist chamber. After three washes with PBS and one wash  
with distilled water, 70  $\mu$ l of test serum obtained  
from one of the panel chimpanzees (identified at the

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left in Table 3) was added to the slides. Each serum sample had been preabsorbed with uninfected liver hybrid cells ( $10^7$  cells per ml of serum) to remove serum factors which tend to bind to the cells non-specifically.

5           The slides containing the added serum were incubated in moist chambers for 90 minutes at room temperature, then washed three times with PBS and once with distilled water.

10           Goat anti-human IgG and IgM conjugated with fluorescein isothiocyanate (FITC-conjugated antibody) was obtained from a commercial source (Zymed Labs) and diluted with PBS to a final concentration of about 1  $\mu$ g antibody/ml. Either anti-IgM or anti-IgG FITC conjugated antibody (70  $\mu$ l) was added to the washed  
15 cells, and the slides were incubated at room temperature for 30 minutes. After washing with PBS and distilled water as above, the slides were mounted with one drop of 50% glycerol in PBS and observed under a fluorescence microscope. The cells were scored for weak (+),  
20 intermediate (++), and strong (+++) fluorescence.

          The first indications of immunofluorescence occurred at about 4-6 weeks after initial cell infection with all three virus sources. The results shown in Table 3 were obtained after 6 weeks infection with  
25 chimpanzee plasma known to contain NANB agent(s). As seen in the righthand columns in Table 4, specific immunofluorescence was observed only with sera from NANB-infected animals, and not with other known sera. The results indicate that (a) the liver hybrid cells are  
30 infectable by NANB virus, (b) the infected hybrid cells are expressing a virus-specific surface antigen which is recognized by NANB serum antibody from chimps with known NANB infection, and (c) an incubation period of between about 4-6 weeks is required for surface antigen

expression. The results shown in Table III were obtained with anti-IgG antibody. No immunofluorescence was observed with the FITC conjugated anti-IgM antibody, as would be expected if the chimp anti-NANB antibodies are  
5 IgG type antibodies.

Table 3

10	<u>Chimpanzee</u>	<u>Disease</u>	<u>Reactivity with liver hybridomas</u>	
			<u>Infected</u>	<u>Uninfected</u>
	A	convalescent HAV	-	-
	B	normal	-	-
	B	acute NANB	+	-
15	C	normal	-	-
	D	normal	-	-
	D	acute NANB	++	-
	E	normal	-	-
	F	convalescent HBV	-	-
20	G	chronic NANB	+++	-
	H	chronic NANB	-	-

25 The human plasma from an individual with acute post-transfusion NANB gave a similar result. After 6 weeks, infected liver hybrids showed specific immunofluorescence with a serum from a NANB-infected chimpanzee, but not with control chimpanzee serum.

30 The NANB-infected hybrid cells were also examined for the presence of NANB virus capable of further cell infection. Infected hybrid cells from above, after 12 weeks incubation, were pelleted and washed three times with PBS. The cells were resuspended in PBS to about  $5 \times 10^6$  cells/ml and sonicated to

clarity. The supernatants (0.5 ml/well) were then inoculated on uninfected hybrids and cultured in the manner described above for cell infection by chimp plasma. After about 4-6 weeks in continuous culture, specific immunofluorescence was observed with chimp NANB serum, but not with serum from uninfected chimps.

Hybrid liver cells infected with plasma from a human with hepatitis B infection showed specific immunofluorescent staining with a goat antiserum to hepatitis B surface antigen six weeks after the hybrid cell culture was infected. Furthermore, the hybrid liver cells secreted hepatitis antigens that can be detected with a commercially available kit that detects hepatitis B surface antigens. This firmly established the ability of the hybrid cells to propagate hepatitis B virus and to secrete hepatitis B specific antigens.

#### Example VI

##### Serial Passage Infection by NANB Virus

One measure of viral infection in cultured cells is by serial passage of the infecting agent from infected to non-infected immortalized culture cells. This example describes the serial passaging of a NANB viral agent from NANB-infected liver cells, designated RO2 and RS1, to uninfected cell line GL424. All of the immortalized cell lines were prepared as in Example III. The RO2 cell line was produced by infection with chimpanzee plasma known to contain NANB viral agent by passage into a second chimpanzee, substantially as described in Example V. The RS1 cell line was produced by infection with the serum from a human who died of NANB hepatitis.

Infection by the NANB viral agent, in serial passage, was confirmed by reacting the newly infected

cells with a NANB-specific human IgG antibody, then with fluorescein-labeled goat anti-human IgG antibody, as in Example V. The antibody was derived from the serum of a human who died of NANB hepatitis, and was specifically  
5 reactive with chimp NANB infected tissue, as assayed in Example V. The antibody was contained in an IgG fraction of the serum prepared conventionally by HPLC.

The following four methods for releasing the viral agent from the two infected cell lines were  
10 employed:

a. The cells were washed 2 times with phosphate buffered saline (PBS). After the second wash, the cell pellet was resuspended in a minimum volume of distilled water (1-2 ml), and the cells were observed  
15 microscopically for cell lysis. When lysis was 90-100% complete, an equal volume of 2X PBS was added, and the lysis suspension was centrifuged at 1500 rpm for 5 minutes. The supernatant was added to the culture medium of uninfected GL424 cells.

20 b. The infected cells were treated with 100 µg/l of mitomycin C for 24 hours. After two washings with PBS, the cells were co-cultivated with uninfected GL424 cells.

c. The infected cells were sonicated with two  
25 15 second bursts, while cooled in an ice bath. Cell lysis was confirmed microscopically. The cell lysis suspension was pelleted as in (a) above and the supernatant added to the culture medium of the uninfected GL424 cells.

30 d. The infected cells were subjected to two freeze/thaw cycles, then pelleted and as in (a) above, before adding the supernatant to uninfected GL424 cells.

In each case, the exposed cells were monitored weekly by surface immunofluorescence for binding to the

above NANB-specific antibody. All four methods showed transmission of the viral agent, as evidenced by immunofluorescence activity, within ten weeks of exposure to the supernatant fractions from each of the two infected cell types. No immunofluorescence activity was observed in control GL424 cells which were exposed to the supernatant from uninfected GL424 cells.

GL424 cells infected with the water-lysis (a) supernatant from infected cells were themselves subject to a water-lysis step, and the supernatant added to the culture medium of uninfected GL424 cells. Within ten weeks, immunofluorescence was observed, evidencing passage of the viral agent from a second to a third generation.

15

#### Example VII

##### NANB-Specific Cell Surface Antigen

The cell lines examined in this example are uninfected line GL424 and infected cell lines RO2 and RS1. Both the RO2 and RS1 lines gave immunofluorescent activity when reacted with the IgG antibodies from NANB-infected human serum and fluorescent-labelled anti-human antibodies, whereas no immunofluorescence was seen with the non-infected GL424 line.

25

Differences in the cell membranes of infected and non-infected cells were examined first because immunofluorescent reactivity is evident on cell membranes in live cell staining with NANB plasma on RO2 and RS1, and secondly because trypsinization of intact GL424 and RO2 cells (which would attack surface proteins) resulted in diminished immunofluorescent reactivity with IgG derived from NANB sera. The protease treatment diminished nonspecific background reactivity (as seen in both GL424 and RO2) and specific

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reactivity, as seen in RO2. The protease sensitivity of RO2 immunoreactivity indicates that the virus-related antigen(s) is proteinaceous or complexed with a protease sensitive moiety. Two-dimensional gel electrophoresis was used in initial efforts to demonstrate protein differences between the infected and non-infected lines. Approximately 500 individual proteins were evident in each pattern. The patterns themselves appeared identical by visual analysis.

A. Membrane Isolation Protocol

GL424, RO2, and RS1 cell lines from above were harvested during log phase, using approximately  $1 \times 10^9$  cells per preparation. Cells were washed three times in Dulbecco's PBS. The pellet was resuspended in 5 ml of a hypotonic solution of 5 mM Tris-HCl (pH 7.4) containing 1 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$ . All of the following steps were done in ice. The cell suspension was placed in a Dounce tissue homogenizer. Cells were disrupted by 10-15 strokes and the homogenate was checked microscopically to determine that most of the cells has ruptured. Immediately after homogenization 2 M sucrose (pH 8) was added to the homogenate to bring the final concentration of sucrose to 0.25 M. Phenylmethylsulfonyl fluoride was added to 0.005% as a protease inhibitor.

The homogenate was centrifuged twice at  $1000 \times g$  to remove nuclei, intact cells, and large mitochondria. The supernatant was centrifuged for two hours at  $85,000 \times g$ . The resulting pellet was resuspended in 1 ml Tris-HCl/ $\text{CaCl}_2$ / $\text{MgCl}_2$  buffer. Aliquots were stored at  $-70^\circ\text{C}$ . These preparations, which contain plasma membranes and small cellular

organelles, were used for all subsequent membrane studies.

B. Immunodot Blots with Membrane Fractions

5           Membrane preps were first examined by  
conventional SDS-PAGE and Western blotting techniques,  
probing with IgG derived from NANB plasma. No specific  
differences in protein profiles were noted among the  
three lines, either in silver-stained gels or in Western  
10   blots probed with NANB IgG.

          Immunodot blots were run using insoluble  
membrane preparations from GL424, RO2, and RS1 at  
concentrations of approximately 100 µg to 0.1 µg per  
well. Blots were probed with normal IgG or NANB IgG  
15   diluted twofold from 1/8 to 1/512. There was  
nonspecific reactivity evident in all reactions of  
membranes with greater than 1 µg/well protein and IgG  
concentrations greater than 1/512 IgG. The results of  
the immunodot blot data are summarized in Table 3 below.

20

25

30

Table 3

## IMMUNODOT BLOT REACTIVITY OF GL525/RO2/RS1 MEMBRANE

5	<u>Cell line</u>	<u>1" Antibody</u>	<u>µg of Membrane per Well</u>		
			<u>10</u>	<u>1</u>	<u>0.1</u>
10	GL424	Normal	+	-	-
		NANB	+	-	-
		none	-	-	-
	RO2	Normal	+	-	-
		NANB	++	+	-
		none	-	-	-
	RS1	Normal	+	-	-
		NANB	++	-	-
		none	-	-	-
15					

a. Results are from Immunoblot Blots probed with 1/512 NANB IgG or normal IgG.

RO2 membrane was reactive with NANB IgG at a concentration of 1 µg/well, while control GL424 was not. A lower amount of nondifferentiated reactivity was evident in all three membrane preparations at 1 µg/well with 1/512 normal IgG. The results from dot blots indicate differences in the immunoreactivity of the NANB-infected line RO2 as compared to uninfected GL424. These results support the finding of antigenic differences indicated by the differential immunofluorescence reactivity of the three cell lines with NANB plasma.

While exemplary embodiments and uses have been described herein, it will be appreciated that the invention encompasses a broad range of human and non-human immortalized tissue cells which can be used in

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stable cell culture and which are infected or infectable  
with a variety of human-infectious viruses.

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IT IS CLAIMED:

1. An immortalized normal human or non-human primate tissue cell line which is infectable by a tissue-specific human virus, comprising  
5 a fusion partner produced by fusing mouse myeloma cells and human B-lymphocytes, and selecting fusion products which show stable human chromosome retention, as evidenced by continued HLA surface antigen  
10 production in culture, and  
fused with the fusion partner, an isolated cell obtained from a human or non-human primate tissue source which is infectable by the selected virus in vivo.
- 15 2. The cell line of claim 1, wherein the fusion partner is produced by fusing mouse myeloma cells and human B-lymphocytes, selecting fusion products which show immunoglobulin secretion and HLA surface antigen  
20 production in culture, treating the selected fusion products with a mutagen, and selecting mutagenized products which retain the ability to produce HLA surface antigen, show no immunoglobulin secretion, and are  
unable to survive in a growth medium which allows growth of a successful product formed by fusing the fusion  
25 partner with such a human cell.
3. The cell line of claim 2, wherein the fusion partner has the characteristics of ATCC NO.HB 8464.  
30
4. The cell line of claim 1, wherein the human cell is selected from the group consisting of hepatocytes, synovial cells, and central nervous system cells.

5. The cell line of claim 4, wherein the human cell is a hepatocyte which is infected by nonAnonB or B hepatitis virus.

5 6. The cell line of claim 5, which has the characteristics of ATCC No.HB 9027.

7. The cell line of claim 4, wherein the isolated cell is from a tissue source which is infected  
10 with the selected virus, and the cell line produced by fusion with the fusion partner is also infected with the selected virus.

8. A method of producing an immortalized normal  
15 human or non-human primate tissue cell line which is infectable by a selected tissue-specific human virus, said method comprising

providing a fusion partner produced by fusing mouse myeloma cells and human B-lymphocytes, and  
20 selecting fusion products which show stable HLA surface antigen production in culture, treating the selected fusion products with a mutagen, and selecting mutagenized fusion products which retain the ability to produce HLA surface antigen, and are unable to survive  
25 in a growth medium which allows growth of a successful product formed by fusing the fusion partner with such a human cell,

obtaining non-lymphocytic cells isolated from a selected human or non-human primate tissue which is  
30 infectable by the selected virus,

fusing the fusion partner with the non-lymphocytic cells obtained, and

selecting fusion products which are infectable with the selected virus.

9. The method of claim 8, for use in producing fused cells which are infected by the selected virus, wherein said selecting includes introducing the selected virus into the fusion products, providing an antiserum  
5 from a human or non-human primate infected with the selected virus, reacting the antiserum with the fusion products, and selecting fused cells which bind virus-specific antibodies present in such antiserum.

10 10. The method of claim 9, wherein said introducing includes infecting the fusion products with the selected virus.

15 11. The method of claim 10, wherein the fusion products are infected with nonAnonB or B hepatitis virus, and the antiserum provided is obtained from a human or chimpanzee having a known nonAnonB or B hepatitis infection, respectively.

20 12. The method of claim 9, wherein said introducing includes obtaining non-lymphocytic cells isolated from a selected human or non-human primate tissue which is infected by the selected virus.

25 13. The method of claim 9, which further includes (a) identifying cell surface antigens associated with specific types of viral agents, and (b) preparing antibodies specific against the cell surface antigens, and (c) using the antibody to isolate  
30 solubilized peptide antigens derived the surface of virus-infected cells.

14. The method of claim 13, wherein the antigens are obtained from cells infected with nonAnonB or B hepatitis virus.

5 15. The method of claim 13, which further includes using the solubilized peptide antigens or segments thereof as a peptide vaccine composition against the virus.

10 16. A method for detecting infection by a tissue-specific human virus which can infect non-lymphocytic tissue cells in a human, with the appearance of serum antibodies which are specific against cell surface antigens present on the infected  
15 cells, said method comprising

providing a surface antigen derived from an immortalized normal human or non-human primate tissue cell which is infected by the tissue-specific human virus and which comprises a fusion partner produced by  
20 fusing mouse myeloma cells and human B-lymphocytes, and selecting fusion products which show stable human chromosome retention, as evidenced by continued HLA surface antigen production in culture and, fused with the fusion partner, an isolated cell obtained from a  
25 human or non-human primate tissue source which is infectable by the tissue-specific virus in vivo.

reacting a blood sample from a human patient with the surface antigen, and

30 detecting the presence of serum antibody which is bound immunospecifically to the surface antigen.

17. The method of claim 16, for detecting infection by nonAnonB virus, wherein the cell surface antigen is derived from an immortalized human liver cell

formed by fusing the fusion partner with human liver cells, and infected with nonAnonB virus.

18. A cell-surface antigen associated  
5 specifically with a human viral agent and derived from  
an immortalized human or non-human primate tissue cell  
line which is infected by the viral agent and which  
comprises a fusion partner produced by fusing mouse  
10 myeloma cells and human B-lymphocytes, and selecting  
fusion products which show stable human chromosome  
retention, as evidenced by continued HLA surface antigen  
production in culture and, fused with the fusion  
partner, an isolated cell obtained from a human or  
non-human primate tissue source which is infectable by  
15 the tissue-specific viral agent in vivo.

19. The antigen of claim 18, wherein the the  
cell surface antigen is derived from an immortalized  
human liver cell formed by fusing the fusion partner  
20 with human liver cells, and infected with nonAnonB virus.

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# INTERNATIONAL SEARCH REPORT

International Application No **PCT/US87/00711**

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) <sup>3</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC  
**IPC(4): C12N 5/00, 7/00, 15/00; C12P 21/00; A61K 39/00; C12Q 1/70**  
**U.S.Cl.: 435/5, 68, 172.2, 240; 424/89; 530/300**

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>4</sup>

Classification System |

Classification Symbols

U.S.	424/89; 436/536, 820; 530/300, 344, 350, 395, 412, 413, 806, 809, 826; 435/5, 7, 68, 70, 172.1, 172.2, 235-237, 239-241, 948 935/89, 90, 92, 93, 95, 96, 99, 100, 102, 106-110
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Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched <sup>5</sup>

Databases: Chemical Abstracts Services Online (File CA, 1967-1987; File Biosis, 1969-1987). See Attachment on Search terms.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>

Category <sup>6</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
P	US, A, 4,634,666 (ENGLEMAN) 6 January 1987, See the entire document.	1-19
Y, P	US, A, 4,634,664 (OESTBERG) 6 January 1987, See the entire document.	1-19
Y, P	US, A, 4,621,053 (SUGIMOTO) 4 November 1986, See the entire document.	1-19
Y	US, A, 4,574,116 (KAPLAN) 4 March 1986, See the entire document.	1-19
Y	US, A, 4,474,893 (READING) 2 October 1984, See the entire document.	1-19
<u>X</u> <u>Y</u>	US, A, 4,542,016 (TREPO) 17 September 1985, See the entire document.	<u>16-19</u> <u>1-15</u>
Y	US, A, 4,491,632 (WANDS) 1 January 1985, See the entire document.	9-14

<sup>15</sup> Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search <sup>2</sup>

Date of Mailing of this International Search Report <sup>2</sup>

29 April 1987

04 JUN 1987

International Searching Authority <sup>1</sup>

Signature of Authorized Officer <sup>19</sup>

ISA/US

Margaret Moskowitz

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X Y	US, A, 4,464,474 (COURSAGET) 7 August 1984, See the entire document.	16-19 1-15
A	US, A, 4,164,566 (PROVOST) 14 August 1979, See the entire document.	1-19
A	US, A, 4,040,905 (PETRICCIANI) 9 August 1977, See the entire document.	1-19

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>10</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>11</sup>

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. **Telephone Practice.**
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, <sup>1a</sup> with indication, where appropriate, of the relevant passages <sup>1b</sup>	Relevant to Claim No <sup>1c</sup>
Y	WO, A 8404325 (INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE) 8 November 1984, See page 23.	1-19
Y	US, A, 4,438,098 (TABOR) 20 March 1984, See the entire document.	15
$\frac{X}{Y}$	US, A, 4,395,395 (TABOR) 26 July 1983, See the entire document.	$\frac{16-19}{1-15}$
Y	Journal of Immunological Methods, Volume 70, Issued May 11, 1984, S.K.H. Fong et al, "Rescue of human monoclonal antibody production from an EBV-transformed B-cell line by fusion to a human-mouse hybridoma," see pages 83-90.	1-19
Y	Journal of Immunological Methods, Volume 80, Issued 25 June 1985,, F.C.M. Van Meel et al, "Human and chimpanzee monoclonal antibodies," see pages 267-276.	1-19
Y	Proceedings of the National Academy of Sciences, USA, Volume 82, Issued September 1985, H.A. Stanley et al, "Monkey-derived monoclonal antibodies against <u>Plasmodium falciparum</u> ," see pages 6272-6275.	1-19
Y	WO, A, 8503946 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE), 12 September 1985, see the entire document.	1-19

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

<u>X</u> <u>Y</u>	US, A, 4,464,474 (COURSAGET) 7 August 1984, See the entire document.	<u>16-19</u> <u>1-15</u>
A	US, A, 4,164,566 (PROVOST) 14 August 1979, See the entire document.	1-19
A	US, A, 4,040,905 (PETRICCIANI) 9 August 1977, See the entire document.	1-19

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>10</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>11</sup>

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone Practice.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

PCT/US87/00711

Attachment to Form PCT/ISA/210, Part VI.

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

- I. Claims 1-14, drawn to a cell line, method of producing the cell line and method of using the cell line; class 435/240, class 435/172.2 and class 530/413.
- II. Claim 15, drawn to a method of using an antigen as a vaccine; class 424/89.
- III. Claims 16 and 17, drawn to an immunoassay; class 435/5.
- IV. Claims 18 and 19, drawn to virus-associated cell surface antigens, class 530/300+.

PCT/US87/00711

Attachment to Form PCT/ISA/210, Part VI. 1.

Telephone approval:

\$420 payment approved by Peter J. Dehlinger on April 24, 1987, for Groups I, II, III, and IV; charge to Deposit Account No. 03-1952. Counsel advised that he has no right to protest for any group not paid for and that any protest must be filed no later than 15 days from the date of mailing of the search report (Form 210). Examiner Margaret Moskowitz.

Reasons for holding lack of unity of invention:

The inventions as defined by Group II (Claim 15) classified in class 424, subclass 89 and Group III (Claims 16 and 17) classified in class 435, subclass 5 are drawn to further alternative uses of the invention of Group I drawn to a cell line, method of preparing the cell line and a method of using the cell line classified in class 435, subclass 240, class 435, subclass 172.2 and class 530, subclass 413 respectively. The invention as defined by Group IV (Claims 18 and 19) is drawn to anti-gens classified in class 530, subclass 300+ which are structurally and functionally distinct from the cell line of Group I.

Time Limit for Filing a Protest

Applicant is hereby given 15 days from the mailing date of this Search Report in which to file a protest of the holding of lack of unity of invention. In accordance with PCT Rule 40.2 applicant may protest the holding of lack of unity only with respect to the group(s) paid for.

PCT/US87/00711

Attachment to Form PCT/ISA/210, Part II.

II. FIELDS SEARCHED SEARCH TERMS:

hepatitis, trioma, hybrid, hepatocyte, liver cell,  
myeloma, fusion, virus, hepatoma; lymphocyte, non-A,  
non-B hepatitis, antigen, culture, vitro, cell surface  
antigen, inventors' names.